c.) Remarks

The specification has been amended for better clarity, as discussed below. No new matter has been added.

Claims 6, 16, 24, 31, 38 and 50 are withdrawn as being directed to a constructively non-elected invention. In response, in order to reduce the issues, these claims are cancelled without prejudice or disclaimer.

Claims 37, 43 and 44 are rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. Applicants previously amended the claims to clarify the accepted relationships among *Saxifragaceae*, *Saxifrage* and *Hydrangea* and, in response, the Examiner stated those "arguments without any evidence are deemed to be speculative [in] nature."

In order to address the Examiner's concerns, Applicants respectfully wish to point out that in the present specification, the plants are classified based on the taxonomy in Makino's Illustrated Flora in Color, 40th edition, Hokuryukan (June 10, 1984) and Makino's New Illustrated Flora of Japan, 1st edition, Hokuryukan (May 10, 1983). See specification page 10, lines 18-23. The genus *Hydrangea* and the genus *Saxifraga* are both classified as belonging to the family *Saxifragaceae* according to this taxonomy.

In 1988, the family *Hydrangeaceae* was separated from the family *Saxifragaceae*, and according to this new taxonomy, the genus *Hydrangea* is classified as belonging to the family *Hydrangeaceae*. The specification has above been amended in conformity therewith.

Claims 37 and 43-44 remain rejected under 35 U.S.C. §103(a) as being unpatentable over Yamahara in combination with Levinson. This rejection is respectfully traversed. Prior to setting forth their bases for traversal, however, Applicants would briefly like to discuss the salient features of the present invention and *inter alia* its patentable nature over the prior art.

As the Examiner is well-aware, the feature of the present invention is the use of an extract obtained by the method according to claims 37 and 43. The use of that particular extract causes excellent effects as evidenced in the present specification.

In support of the rejection, the Examiner states that Yamahara teaches on page 2, paragraph 2 of English translation that the methanol extract of *Hydrangeae Dulcis Folium* itself has strong radical eliminating effect and inhibitory effect on oxidation of lipids and not just the crude drug phyllodulcin. According to the Examiner,

> <u>if liver peroxidation were inhibited, irrespective of the cause of the lipid peroxidation, the liver function would be protected</u>. Page 4 of the Office Action at lines 7-8 (emphasis added).

However, there is no recognized correlation between the inhibitory activity on lipid peroxidation and the protective potency against liver injury. Accordingly, liver injuries such as hepatonecrosis cannot be protected simply by inhibiting lipid peroxidation, as propounded by the Examiner to support obviousness. Indeed, this is explicitly shown in Suzuki et al. (*Yakugaku Zasshi*, 110(9), 697-701 (1990)), the original and an English translation of which are both enclosed herewith at Tabs A and B. The Examiner's attention is respectfully invited to the underlined portion in the Results and discussion of the reference (pages 3-5 of the translation).

As discussed in Suzuki, fourteen representative commercial antioxidants were evaluated for protective effect on liver injury at their maximum administrable dosages. In distinct contrast to the Examiner's assertions noted above, potency was found only for a very few, and worsening of liver injury was evidenced for several. Moreover, as stated at page 3, lines 29-34,

> [a]s a result of administering these antioxidants to normal rats, no significant difference was found between the group treated with any of them and the non-treated group and thus, it was confirmed that there is no direct influence of the antioxidants on the determined value of the serum components. (Emphasis added.)

Additionally, these data confirm that liver injury cannot be prevented simply by inhibiting increased lipid peroxide content (see page 4). For instance, BHA (butylated hydroxyanisole) is highly antioxidative but provides no liver injury protection, while cysteine (which is only very mildly antioxidative) provides excellent liver injury protection (see page 5).

This knowledge belies the Examiner's bases of rejection and plainly fail to suggest the unexpected results obtained by the present invention.

Specifically, example 16 relates to the inhibiting activity of extracts of *Hydrangeae Dulcis Folium* on D-galactoseamine-induced rat hepatopathy using the feed of Example 10 containing 1% freeze-dried power of Example 5, which is with the extraction method of the present invention. Similarly, example 22 relates to the building inhibiting activity of an ethanol extract of the residue of a water extract of *Hydrangeae Dulcis Folium* on alcohol/LPS-induced rat hepatopathy using the feed of Example 20

containing 1% freeze-dried powder of Example 5 obtained with the extraction method of the present invention.

Both of these examples show that the inhibiting activity of the extract obtained by the method of the present invention is several fold higher than the inhibiting activity of an extract obtained from the same plants using conventional methods.[1]

In view of the above amendments and remarks, Applicants submit that all of the Examiner's concerns are now overcome and the claims are now in allowable condition. Accordingly, reconsideration and allowance of this application is earnestly solicited.

Claims 37, 43 and 44 remain presented for continued prosecution.

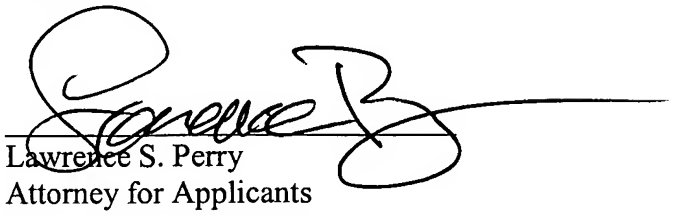
---

[1] As shown in Table 1 of Example 16, GTP activities (%) of the feeds of Examples 7-9 (containing the freeze-dried powders obtained by methods other than the present invention, e.g., powders produced in Example 1, 2 and 4) are 30.5-49.8. In contrast, GTP activity (%) of the feed of Example 10 (containing freeze-dried powder obtained according to the present invention) is 14.3. The result indicates that the hepatopathy-inhibiting activity of the feed containing the freeze-dried powder obtained by the method of the present invention is vastly superior higher than those of the controls.

Similarly, table 5 of Example 22 shows that the serum GPT and GOT activities which are indications of liver function disorder were as low as 10.8% and 7.7% of those of the control group using the feed produced in Comparative Example 2. These results too indicate that hepatopathy-inhibiting activity of the feed containing the extract obtained by the method of the present invention is remarkably higher than the controls.

Applicants' undersigned attorney may be reached in our New York office by telephone at (212) 218-2100. All correspondence should continue to be directed to our below listed address.

Respectfully submitted,



Lawrence S. Perry
Attorney for Applicants
Registration No. 31,865

FITZPATRICK, CELLA, HARPER & SCINTO
30 Rockefeller Plaza
New York, New York 10112-3801
Facsimile: (212) 218-2200

LSP\ac

NY_Main 575099_1

薬学雑誌
YAKUGAKU ZASSHI
110(9) 697—701 (1990)

抗酸化物の実験的肝障害防護効果[1]

鈴木 真,[a] 熊沢紀子,[a] 太田節子,[a] 鴨川 旭,[b] 篠田雅人*,[a]
星薬科大学,[a] 聖マリアンナ医科大学[b]

Protective Effects of Antioxidants on Experimental Liver Injuries[1]

Makoto SUZUKI,[a] Noriko KUMAZAWA,[a] Setsuko OHTA,[a]
Asahi KAMOGAWA[b] and Masato SHINODA*,[a]
Faculty of Pharmaceutical Science, Hoshi University,[a] 2-4-41, Ebara, Shinagawa-ku, Tokyo, 142, Japan and St. Marianna University School of Medicine,[b] 2-16-1, Sugao, Miyamae-ku, Kawasaki, 213, Japan

(Received March 13, 1990)

Protective effects of 14 kinds of antioxidant on liver injury induced by carbon tetrachloride (CCl_4) were investigated in terms of serum enzyme activities and bilirubin concentration. Consequently, the significant protective effects were found in sesamol, ellagic acid, cysteamine and cysteine. These antioxidants clearly decreased the lipid peroxide in the liver tissue. The protective effects on CCl_4-induced liver injury *in vivo* were independent of the inhibitory activities on lipid peroxidation in hepatic mitochondria fraction *in vitro*.

Keywords——antioxidant; liver injury protection; lipid peroxide; carbon tetrachloride; sesamol; tocopherol

四塩化炭素 (CCl_4) による肝障害の主因の 1 つは脂質過酸化反応であると考えられていることから,[2,3] CCl_4 肝障害に対する抗酸化物の効果が検討されている.[4-9] *dl*-α-Tocopherol などいくつかの抗酸化物は CCl_4 による肝の脂質過酸化反応を抑制することが報告されているが,[7,8] 血清逸脱酵素を指標とした肝障害に対する抗酸化物の効果を検討しているものは少ない. また実験動物に CCl_4 を 1 回投与した後の血清逸脱酵素活性は組織学的に評価した肝障害の程度と強い相関があることが知られている.[9] 本実験では, 代表的な抗酸化物 14 種が血清成分の変動を指標とした CCl_4 肝障害を防護するか否かを検討した. また, *in vitro* の系における脂質過酸化抑制活性を測定し, 抗酸化活性と実験的肝障害防護効力との関係を検討した.

実 験 の 部

使用薬物 Cysteine は Sigma 社製を, sesamol, ellagic acid は Aldrich 社製を, 他の薬物は和光純薬工業製を用いた.

実験的肝障害防護効力試験 実験は, 著者らがすでに報告した方法[10]で行った. CCl_4 0.3 ml/kg をオリブ油溶液として腹腔内投与し, 投与 24 h 後に, 腹部下行大静脈より採血した. 血清成分の glutamate oxalacetate transaminase (GOT), glutamate pyruvate transaminase (GPT) は Karmen 法,[11] lactate dehydrogenase (LDH) は Wacker 法,[12] leucine aminopeptidase (LAP) は Nagel 法,[13] 総ビリルビン (T-BIL) と直接ビリルビン (D-BIL) は Jendrassik 法[14]により, 生化学自動分析装置 (TBA-380, 東芝メディカル) で測定した. 試料は水又は 5% Tween 80 で懸濁させ障害誘発薬物投与 30 min 前に腹腔内投与した.

肝組織中の過酸化脂質量の測定 CCl_4 肝障害効力試験時に, 採血後肝臓を摘出し, 5% ホモジネート液を作製し, チオバルビツール酸 (TBA) 法[15,16]により過酸化脂質量を測定した. 過酸化脂質量は, 肝重量 1 g 当たりのマロンジアルデヒド (MDA) 量として表した.

肝障害防護効力の表示[10] 肝障害防護効力は各測定値の平均を次式に代入し算出した.

$$\text{防護効力 (\%)} = \frac{\text{誘発薬物投与群} - (\text{試料} + \text{誘発薬物})\ \text{投与群}}{\text{誘発薬物投与群} - \text{無処置群}} \times 100$$

TABLE I. Protective Effects of Antioxidants on Liver Injury Induced by CCl_4

Sample	Molecular weight	Dose (mmol/kg)	GOT (IU/l)	GPT (IU/l)	LDH (IU/l)	LAP (IU/l)	T-BIL (mg/dl)	D-BIL (mg/dl)
Nontreated			123± 18	28± 6	1363± 302	21± 2	0.28±0.04	0.15±0.02
CCl_4 control			9852±2785	1804±407	16584± 3884	60± 9	1.05±0.27	0.57±0.13
L-Ascorbic acid	198.11	1	5813±1828[a]	1427±398	13103± 4400	49± 4	0.99±0.28	0.50±0.12
			(41.5)	(21.2)	(22.9)	(28.2)	(7.8)	(16.7)
Isoascorbic acid	176.12	1	7353±3666	1625±982	12123± 6108	49± 8	0.90±0.40	0.45±0.20
			(25.7)	(10.1)	(29.3)	(28.2)	(19.5)	(28.6)
Sesamol	138.12	1	628± 36[b]	200± 50[b]	1570± 250[b]	20± 2[b]	0.41±0.10[b]	0.22±0.06[b]
			(94.8)	(90.3)	(98.6)	(102.6)	(83.1)	(83.3)
Gallic acid (hydrate)	188.14	1	6535±2070	1753±396	15090± 4222	48±10	1.03±0.14	0.52±0.08
			(34.1)	(2.9)	(9.8)	(30.8)	(2.6)	(11.9)
Ferulic acid	194.19	1	8955±2034	1933±436	19418± 3862	53± 6	1.39±0.20	0.67±0.12
			(9.2)	(− 7.3)	(−18.6)	(17.9)	(−44.1)	(−23.8)
Ellagic acid (dihydrate)	338.22	0.12	3167±1448[b]	729±264[b]	6680± 3180[b]	33±10[b]	0.67±0.26[a]	0.40±0.12[a]
			(68.7)	(60.5)	(65.1)	(69.2)	(49.4)	(40.5)
BHT	220.36	1	10083±1518	2132±374	23135± 5246[b]	56± 8	1.60±0.14[b]	0.79±0.12[b]
			(−2.4)	(−18.5)	(−43.0)	(10.3)	(−71.4)	(−52.4)
BHA	180.25	0.5	8380±4186	2059±850	23458±15052	66±16	1.10±0.50	0.59±0.24
			(15.1)	(−14.4)	(−45.2)	(−15.4)	(− 6.5)	(4.8)
dl-α-Tocopherol	430.72	1	9463±2458	1509±324	16633± 2012	61± 2	1.28±0.20	0.76±0.16
			(4.0)	(16.6)	(− 0.3)	(− 2.6)	(−29.9)	(−45.2)
dl-α-Tocopherol acetate	472.75	1	7860±2874	1549±552	18780± 6328	56± 8	1.27±0.54	0.65±0.24
			(20.5)	(14.4)	(−14.4)	(10.3)	(−28.6)	(−19.0)
Vitamin K_1	450.71	1	6528±4498	1559±750	15750± 8256	56±14	1.14±0.56	0.61±0.24
			(34.2)	(13.8)	(5.5)	(10.3)	(11.7)	(− 9.5)
Cysteamine (hydrochloride)	113.60	2	1160± 602[b]	281±140[b]	2103± 1420[b]	29± 4[b]	0.36±0.10[b]	0.22±0.08[b]
			(89.3)	(85.8)	(95.1)	(79.5)	(89.6)	(83.3)
Cysteine	121.20	8	1235± 674[b]	327±124[b]	1725± 626[b]	32± 8[b]	0.30±0.08[b]	0.21±0.08[b]
			(88.6)	(83.2)	(97.6)	(71.8)	(97.4)	(85.7)
Linoleic acid	302.43	0.08	4998±1678[a]	1482±584	9573± 3924	53±12	0.81±0.28	0.35±0.06[a]
			(49.9)	(18.1)	(46.1)	(17.9)	(31.2)	(52.4)

Each value is the mean±S.D. of 4 rats (only CCl_4-treated group is 20 rats). Values in parentheses indicate protective potencies.

$$\text{Protective potency (\%)} = \frac{(CCl_4 \text{ group}) - (\text{Sample} + CCl_4 \text{ group})}{(CCl_4 \text{ group}) - (\text{Nontreated group})} \times 100$$

Significantly different from CCl_4-treated control group. a) $p<0.05$, b) $p<0.01$ (Student's t-test).

試料の効果は，誘発薬物のみの投与群に対する（試料＋誘発薬物）投与群の各測定値の差の有意性を Student's *t*-test により検定した．

肝ミトコンドリア脂質過酸化抑制活性の測定　実験は，Okuda ら[17] の方法に従って行った．

結果及び考察

1. CCl_4 肝障害に対する抗酸化物の防護効果

抗酸化物 14 種について，CCl_4 肝障害防護効力を検討した (Table I)．投与量は毒性を考慮し，すべて投与可能な最大量で検討した結果，すでに効力の認められている cysteamine,[10] cysteine[16] のほかに，sesamol, ellagic acid にすべての項目で有意な効力が認められた．しかし，butyl hydroxytoluene (BHT), *dl*-α-tocopherol では悪化する傾向がみられた．また，これらの抗酸化物を正常ラットに投与した結果，いずれも無処置群との間に有意な差は認められず血清成分測定値に対する直接的な影響はないことを確認した (Table II)．

Sesamol はゴマ油の中に含まれている抗酸化物であり，強い抗酸化活性を有するといわれているものである．本実験では，著しく強い CCl_4 肝障害防護効力が認められた．Ellagic acid は，エラジタンニンが加水分解して生ずるポリフェノールであり，抗変異原性,[19,20] 抗発ガン性[21] があると言われている．本実験では溶解性が低く懸濁状態で投与しているため，更に溶媒などの検討が必要であると思われる．*In vivo* で有効であった 4 種の薬物は，いずれも CCl_4 による肝組織中の過酸化脂質含量の上昇を有意に抑制した (Table III)．しかし，同様に過酸化脂質含量の上昇を有意に抑制した *dl*-α-tocopherol, *dl*-α-tocopherol acetate の 2 種 (Table III) は，血清成分の変動を指標とした CCl_4 肝障害には無効であった (Table I)．これらのことから，CCl_4 による肝組織中の過酸化脂質含量の上昇を抑制しただけでは，血清成分の変動を指標とした CCl_4 肝障害を防護できないと考えられる．しかし *dl*-α-tocopherol については Yoshikawa ら[8] が，Wistar 系雌性ラットに α-tocopherol を飼料に混ぜ 4ヶ月間摂取させた後，CCl_4 を 1 回腹腔内投与することにより血清中 GPT を指標とした CCl_4 肝障害を防護することを認めており，*dl*-α-tocopherol は実験条件によっては血清成分の変動を指標とした CCl_4 肝障害も防護すると思われる．

2. 肝ミトコンドリアにおける脂質過酸化抑制効果

In vitro の系における抗酸化物の脂質過酸化抑制活性の測定には，リノール酸の空気酸化を用いる方法,[18] リボヤシゲナーゼにより脂質過酸化を起こさせる方法,[22] 肝ミクロソーム又は肝ミトコンドリアを用いる方法[17] などすでに様々な方法が行われている．本実験では，薬物代謝酵素系に対する各薬物の影響を考慮し，肝ミトコンドリアを用いた方法を行った (Table IV)．その結果，butyl hydroxyanisole (BHA) と sesamol に *dl*-α-tocopherol

Table II. Influence of Only Antioxidants Administration on Serum Enzyme Activities

Sample	Molecular weight	Dose (mmol/kg)	GOT (IU/l)	GPT (IU/l)	LDH (IU/l)	LAP (IU/l)	T-BIL (mg/dl)	D-BIL (mg/dl)
Nontreated			130±111	18±3	434± 61	19±3	0.23±0.09	0.13±0.09
L-Ascorbic acid	198.11	1	119± 16	21±4	807±640	21±4	0.21±0.06	0.10±0.04
Sesamol	138.12	1	126± 11	22±2	835± 91	20±2	0.20±0.02	0.11±0.02
Gallic acid (hydrate)	188.14	1	97± 17	17±5	637±140	16±3	0.22±0.05	0.11±0.03
Ferulic acid	194.19	1	111± 16	16±3	817± 69	19±3	0.22±0.03	0.12±0.03
Ellagic acid (dihydrate)	338.22	0.12	117± 3	15±3	560±159	15±3	0.21±0.03	0.10±0.03
BHT	220.36	1	117± 36	17±3	448± 99	20±3	0.24±0.12	0.12±0.03
BHA	180.25	0.5	145± 69	20±5	545±381	16±3	0.25±0.03	0.14±0.05
dl-α-Tocopherol acetate	472.75	1	98± 16	19±3	462±152	20±3	0.18±0.03	0.08±0.03
Cysteine	121.20	8	124± 16	23±5	688±282	19±3	0.20±0.05	0.08±0.03
Linoleic acid	302.43	0.08	102± 33	16±3	547±151	19±3	0.19±0.02	0.09±0.03

Each value is the mean±S.D. of 3 rats.

TABLE III. Protective Effects of Antioxidants on Lipid Peroxidation Induced by CCl_4

Sample	Molecular weight	Dose (mmol/kg)	Lipid peroxide MDA nmol/g tissue
Nontreated			133± 74
CCl_4 control			4312±1673
L-Ascorbic acid	198.11	1	4444±1636 (−3.2)
Isoascorbic acid	176.12	1	2403±1160[a] (45.7)
Sesamol	138.12	1	212± 142[b] (98.1)
Gallic acid (hydrate)	188.14	1	3695±1318 (14.8)
Ferulic acid	194.19	1	4001± 302 (7.4)
Ellagic acid (dihydrate)	338.22	0.12	1511±1632[a] (67.0)
BHT	220.36	1	3233± 234 (25.8)
BHA	180.25	0.5	3455± 540 (20.5)
dl-α-Tocopherol	430.72	1	336± 84[b] (95.1)
dl-α-Tocopherol acetate	472.75	1	148± 46[b] (99.6)
Vitamin K_1	450.71	1	2445±1008[a] (44.7)
Cysteamine (hydrochloride)	113.60	2	173± 93[b] (99.0)
Cysteine	121.20	8	104± 32[b] (100.7)
Linoleic acid	302.43	0.08	227± 18[b] (97.8)

Each value is the mean±S.D. of 4 rats (only CCl_4-treated group is 20 rats). Values in parentheses indicate protective potencies.

$$\text{Protective potency (\%)} = \frac{(CCl_4 \text{ group}) - (\text{Sample} + CCl_4 \text{ group})}{(CCl_4 \text{ group}) - (\text{Nontreated group})} \times 100$$

Significantly different from CCl_4-treated control group. *a*) $p<0.05$, *b*) $p<0.01$.

TABLE IV. Inhibition of Lipid Peroxidation of Liver Mitochondoria Fraction Induced by Ascorbic Acid Plus ADP-Fe^{3+}

Sample[a]	Molecular weight	IC_{50}[b] (M)	IC_{50}[b] (μg/ml)
BHA	180.25	3.94×10^{-6}	0.71
Sesamol	138.12	8.83×10^{-6}	1.22
dl-α-Tocopherol	430.72	1.61×10^{-5}	6.93
Gallic acid (hydrate)	188.14	5.43×10^{-5}	10.23
Ellagic acid (dihydrate)	338.22	6.16×10^{-4}	208.45
Cysteamine (hydrochloride)	113.60	6.35×10^{-4}	72.12
Linoleic acid	302.43	9.05×10^{-4}	273.56
Ferulic acid	194.19	9.09×10^{-4}	176.54
dl-α-Tocopherol acetate	472.75	1.17×10^{-3}	554.62
Cysteine	121.20	4.44×10^{-3}	537.98
Vitamin K_1	450.71	$>2.22\times10^{-3}$	>1000.00

a) Each sample was dissolved in Krebs-Ringer phosphate buffer or 5% Tween 20. *b*) 50% Inhibitory concentration.

よりも強い脂質過酸化抑制効果がみられ，また vitamin K_1 には効力が認められなかった．高い抗酸化能を示した BHA が CCl_4 肝障害には無効であり，一方高い CCl_4 肝障害防護効力を示した cysteine の脂質過酸化抑制効力は他の化合物に比べて弱いことなどから，*in vitro* での肝ミトコンドリアにおける脂質過酸化抑制活性と *in vivo* での CCl_4 肝障害防護効力との相関性は低いと思われる．

以上すべての結果から考察すると，血清成分の変動により評価される肝壊死のような CCl_4 肝障害は，CCl_4 による脂質過酸化を抑制しただけでは防護できないことが示唆される．つまり脂質過酸化以外の要因が関与している可能性が考えられるため，CCl_4 が血中に酵素を逸脱させる機構についてさらに検討する必要があると思われる．

引用文献及び注

1) 本研究の一部は日本薬学会第 109 年会で発表，名古屋，1989 年 4 月．
2) R. O. Recknagel, *Pharmacol. Rev.*, 19, 145 (1967).
3) E. A. Glende, A. M. Hruszkewycz, R. O. Recknael, *Biochem. Pharmacol.*, 25, 2163 (1976).
4) E. C. Ferreyra, J. A. Castro, M. I. D. Gomez, N. D. Acosta, C. R. Castro, O. M. Fenos, *Toxicol. Appl. Pharmacol.*, 32, 504 (1975).
5) R. J. Ruch, J. E. Klaauning, N. E. Shultz, *Envir. Health Perspect.*, 69, 301 (1986).
6) A. K. Ghoshal, *Exp. Mol. Pathol.*, 25, 202 (1976).
7) A. L. Tapple, *Ann. N. Y. Acad. Sci.*, 355, 18 (1980).
8) T. Yoshikawa, Y. Furukawa, M. Murakami, *Digestion*, 25, 222 (1982).
9) 長瀬すみ，田中寿子，“実験動物の臨床生化学データ—病理組織像との関連—，”ソフトサイエンス社，1976, pp. 220—235.
10) 太田節子，熊坂昌子；篠田雅人，薬誌，105, 866 (1985).
11) A. Karmen, F. Wroblevski, J. S. LaDue, *J. Clin. Invest.*, 34, 131 (1955).
12) W. E. C. Wacker, D. D. Ulmer, B. L. C. Vallee, *New. England. J. Med.*, 255, 449 (1956).
13) W. Thefeld, H. Hoffmeister, E. W. Busch, P. U. Koller, J. Vollmar, *Deut. Med. Wochenschr*, 99, 343 (1974).
14) L. Jendrassik, R. A. Cleghorm, *Biochem. Z.*, 289, 1 (1936).
15) H. Tanizawa, Y. Sazuka, Y. Takino, *Chem. Pharm. Bull.*, 29, 2910 (1981).
16) H. Tanizawa, Y. Sazuka, A. Komatsu, S. Toda, Y. Takino, *Chem. Pharm. Bull.*, 31, 4139 (1983).
17) T. Okuda, Y. Kimura, T. Yoshida, T. Hatano, H. Okuda, S. Arichi, *Chem. Pharm. Bull.*, 31, 1625 (1983).
18) E. C. Ferreyra, O. M. Fenos, A. S. Bernacchi, C. R. Castro, J. A. Castro, *Toxicol. Appl. Pharmacol.*, 42, 513 (1977).
19) A. W. Wood, M. T. Huang, R. L. Chang, H. L. Newmark, R. E. Lehr, H. Yagi, J. M. Sayer, D. M. Jerina, A. H. Conney, *Proc. Natl. Acad. Sci. U.S.A.*, 79, 5513 (1982).
20) J. M. Sayer, H. Yagi, A. W. Wood, A. H. Conney, D. M. Jerina, *J. Am. Chem. Soc.*, 104, 5562 (1982).
21) H. Mukhtal, M. Das, B. J. Del Tito, Jr., D. Bickers, *Biochem. Biophys. Res. Commun.*, 119, 751 (1984).
22) 藤田勇三郎，上原都意，森本泰子，中島真由美，波多野力，奥田拓男，薬誌，108, 129 (1988).

YAKUGAKU ZASSHI
110 (9) 697-701 (1990)

Protective Effects of Antioxidants on Experimental Liver Injuries[1)]

Makoto SUZUKI,[a] Noriko KUMAZAWA,[a] Setsuko OHTA,[a]
Asahi KAMOGAWA[b] and Masato SHINODA*,[a]

*Faculty of Pharmaceutical Science, Hoshi University,[a]
2-4-41, Ebara, Shinagawa-ku, Tokyo, 142, Japan
and St. Marianna University School of
Medicine,[b] 2-16-1, Sugao, Miyamae-ku,
Kawasaki, 213, Japan*

(Received March 13, 1990)

Protective effects of 14 kinds of antioxidant on liver injury induced by carbon tetrachloride (CCl_4) were investigated in terms of serum enzyme activities and bilirubin concentration. Consequently, the significant protective effects were found in sesamol, ellagic acid, cysteamine and cysteine. These antioxidants clearly decreased the lipid peroxide in the liver tissue. The protective effects on CCl_4-induced liver injury *in vivo* were independent of the inhibitory activities on lipid peroxidation in hepatic mitochondria fraction *in vitro*.

Keywords - antioxidant; liver injury protection; lipid peroxide; carbon tetrachloride; sesamol; tocopherol

As lipid peroxidation is considered to be one of the main causes for liver injury induced by carbon tetrachloride (CCl_4),[2),3)] the effects of antioxidants on CCl_4-induced liver injury have been investigated.[4-6)] Although some antioxidants including *dl*-α-tocopherol have been reported to inhibit lipid peroxidation of liver induced by CCl_4,[7,8)] there are not many

reports that investigate the effects of antioxidants on liver in terms of serum enzymes. It is known that serum enzyme activities after administration of CCl_4 to experimental animals once have a strong correlation with the degree of histologically determined liver injury.[9] In this experiment, we investigated whether or not 14 kinds of typical antioxidants protect CCl_4-induced liver injury in terms of variation in the serum components. We also investigated relation between antioxidative activity and protective potency against experimental liver injury by determining inhibitory activity on lipid peroxidation *in vitro*.

Experiment

Substances used Cysteine produced by Sigma, and sesamol and ellagic acid produced by Aldrich were used. Other drugs used are the products of Wako Junyaku.

Test on the protective potency against experimental liver injury Experiments were carried out according to the method already reported by the present authors.[10] An olive oil solution of 0.03 ml/kg of CCl_4 was intraperitonealy administered. After 24 hours of administration, blood was collected from abdominal inferior vena cava. Of the serum components, glutamate oxalacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) were determined by the method of Karmen,[11] lactate dehydrogenase (LDH) by the method of Wacker,[12] leucine aminopeptidase (LAP) by the method of Nagel,[13] and total bilirubin (TBIL) and direct bilirubin (DBIL) by the method of Jendrassik[14] using a biochemical autoanalyzer (TBA-380, Toshiba Medical). The samples were suspended in water or 5% Tween 80 and intraperitoneally administered 30 minutes prior to the administration of each injury inducer.

Determination of the amount of lipid peroxide in the liver tissue At the time test was carried out on the protective potency against liver injury induced by CCl_4, the liver was excised after the collection of blood, its 5% homogenate solution was prepared, and the amount of lipid peroxide was determined by the thiobarbituric acid (TBA) method.[15,16] The amount of lipid peroxide was expressed

as the amount of malondialdehyde (MDA) per 1 g by weight of the liver.

Indication of the protective potency against liver injury[10] Protective potency against liver injury was calculated by applying each mean value of the determined values to the following equation:

$$\text{Protective potency (\%)} = \frac{\text{Inducer-administered group} - (\text{Sample} + \text{Inducer})\text{-administered group}}{\text{Inducer-administered group} - \text{Non-treated group}} \times 100$$

As regards the effect of the samples, significance of the difference in the determined values between the (sample + Inducer)-administered group and the only inducer-administered group was tested according to Student's t-test.

Determination of inhibitory activity on lipid peroxidation in hepatic mitochondria Experiments were carried out according to the method of Ohta et al.[17]

Results and Discussion

1. Protective effect of antioxidants on liver injury induced by CCl_4

Protective effect on liver injury induced by CCl_4 was investigated with respect to 14 kinds of antioxidants (Table I). Taking toxicity into consideration, investigation was carried out using maximum administrable dose for all the antioxidants. As a result, significant potency was found with sesamol and ellagic acid in addition to cysteamine[10] and cysteine[18] with which the potency has already been found with respect to all the items. However, a worsening tendency was observed with butyl hydroxytoluene (BHT) and *dl*-α-tocopherol. As a result of administering these antioxidants to normal rats, no significant difference was found between the group treated with any of them and the non-treated group and thus, it was confirmed that there is no direct influence of the antioxidants on the determined values of the serum components (Table II).

Sesamol is an antioxidant contained in sesame oil and is said to have a strong antioxidative activity. In this experiment, a remarkably strong protective potency of sesamol was found against liver injury induced by CCl_4. Ellagic acid is a polyphenol formed by hydrolysis of ellagitannin and is said to have antimutagenicity[19,20] and anticarcinogenicity.[21] In this experiment, this substance was administered in an emulsified state as it is poorly soluble. Further investigation on suitable solvents may be necessary. Any of the 4 kinds of substances which were effective *in vivo* significantly inhibited increase of the lipid peroxide content in the liver tissue induced by CCl_4 (Table III). However, 2 kinds of substances *dl*-α-tocopherol and *dl*-α-tocopherol acetate which likewise inhibited increase of the lipid peroxide content (Table III) were ineffective on CCl_4-induced liver injury in terms of variation in the serum components (Table I). From these findings, it is considered that CCl_4-induced liver injury in terms of the serum components cannot be protected only by inhibiting the increase of lipid peroxide content in the liver tissue induced by CCl_4. However, as to *dl*-α-tocopherol, Yoshikawa et al.[8] found that α-tocopherol protected CCl_4-induced liver injury in terms of GTP in serum by administering the substance as a feed mixture to female rats of Wistar strain for 4 months followed by intraperitoneal administration of CCl_4 once. Thus, *dl*-α-tocopherol is considered to protect CCl_4-induced liver injury in terms of variation in the serum components depending upon the experimental conditions.

2. Inhibitory effect on lipid peroxidation in hepatic mitochondria

For the determination of inhibitory activity of antioxidants on *in vitro* lipid peroxidation, various methods including the method using air oxidation of linoleic acid,[16] the method inducing lipid peroxidation by lipoxygenase[22] and the method using hepatic microsome or hepatic mitochondria[17] are carried out. In this experiment, we carried out the method using hepatic mitochondria (Table IV), considering the influence of the test substances on the drug metabolism enzyme systems. As a result, a stronger inhibitory effect was found with butyl hydroxyanisole (BHA) and

sesamol than with *dl*-α-tocopherol. Further, no effect was found with vitamin K_1. From the fact that BHA which showed high antioxidative ability is ineffective on CCl_4-induced liver injury whereas cysteine that showed high protective potency against CCl_4-induced liver injury has a weak inhibitory potency against lipid peroxidation compared with other compounds, correlation between inhibitory activity on lipid peroxidation in hepatic mitochondria *in vivo* and protective potency against CCl_4-induced liver injury *in vitro* may be rather low.

Considering from all of the above results, it is suggested that CCl_4-induced liver injuries such as hepatonecrosis that are estimated by variation in the serum components cannot be protected only by inhibiting lipid peroxidation induced by CCl_4. That is to say, CCl_4-induced liver injuries may involve a factor other than lipid peroxidation. So, it may be necessary to further investigate a mechanism in which CCl_4 deviates enzymes into blood.

TABLE I. Protective Effects of Antioxidants on Liver Injury Induced by CCl_4

Sample	Molecular weight	Dose (mmol/kg)	GOT (IU/l)	GPT (IU/l)	LDH (IU/l)	LAP (IU/l)	T-BIL (mg/dl)	D-BIL (mg/dl)
Nontreated			123± 18	28± 6	1363± 302	21± 2	0.28±0.04	0.15±0.02
CCl_4 control			9852±2785	1804±407	16584± 3884	60± 9	1.05±0.27	0.57±0.13
L-Ascorbic acid	198.11	1	5813±1828[a]	1427±398	13103± 4400	49± 4	0.99±0.28	0.50±0.12
			(41.5)	(21.2)	(22.9)	(28.2)	(7.8)	(16.7)
Isoascorbic acid	176.12	1	7353±3666	1625±982	12123± 6108	49± 8	0.90±0.40	0.45±0.20
			(25.7)	(10.1)	(29.3)	(28.2)	(19.5)	(28.6)
Sesamol	138.12	1	628± 36[b]	200± 50[b]	1570± 250[b]	20± 2[b]	0.41±0.10[b]	0.22±0.06[b]
			(94.8)	(90.3)	(98.6)	(102.6)	(83.1)	(83.3)
Gallic acid (hydrate)	188.14	1	6535±2070	1753±396	15090± 4222	48±10	1.03±0.14	0.52±0.08
			(34.1)	(2.9)	(9.8)	(30.8)	(2.6)	(11.9)
Ferulic acid	194.19	1	8955±2034	1933±436	19418± 3862	53± 6	1.39±0.20	0.67±0.12
			(9.2)	(− 7.3)	(−18.6)	(17.9)	(−44.1)	(−23.8)
Ellagic acid (dihydrate)	338.22	0.12	3167±1448[b]	729±264[b]	6680± 3180[b]	33±10[b]	0.67±0.26[a]	0.40±0.12[a]
			(68.7)	(60.5)	(65.1)	(69.2)	(49.4)	(40.5)
BHT	220.36	1	10083±1518	2132±374	23135± 5246[b]	56± 8	1.60±0.14[b]	0.79±0.12[b]
			(−2.4)	(−18.5)	(−43.0)	(10.3)	(−71.4)	(−52.4)
BHA	180.25	0.5	8380±4186	2059±850	23458±15052	66±16	1.10±0.50	0.59±0.24
			(15.1)	(−14.4)	(−45.2)	(−15.4)	(− 6.5)	(4.8)
dl-α-Tocopherol	430.72	1	9463±2458	1509±324	16633± 2012	61± 2	1.28±0.20	0.76±0.16
			(4.0)	(16.6)	(− 0.3)	(− 2.6)	(−29.9)	(−45.2)
dl-α-Tocopherol acetate	472.75	1	7860±2874	1549±552	18780± 6328	56± 8	1.27±0.54	0.65±0.24
			(20.5)	(14.4)	(−14.4)	(10.3)	(−28.6)	(−19.0)
Vitamin K_1	450.71	1	6528±4498	1559±750	15750± 8256	56±14	1.14±0.56	0.61±0.24
			(34.2)	(13.8)	(5.5)	(10.3)	(11.7)	(− 9.5)
Cysteamine (hydrochloride)	113.60	2	1160± 602[a]	281±140[b]	2103± 1420[b]	29± 4[b]	0.36±0.10[b]	0.22±0.08[b]
			(89.3)	(85.8)	(95.1)	(79.5)	(89.6)	(83.3)
Cysteine	121.20	8	1235± 674[b]	327±124[b]	1725± 626[a]	32± 8[b]	0.30±0.08[b]	0.21±0.08[b]
			(88.6)	(83.2)	(97.6)	(71.8)	(97.4)	(85.7)
Linoleic acid	302.43	0.08	4998±1678[a]	1482±584	9573± 3924	53±12	0.81±0.28	0.35±0.06[a]
			(49.9)	(18.1)	(46.1)	(17.9)	(31.2)	(52.4)

Each value is the mean±S.D. of 4 rats (only CCl_4-treated group is 20 rats). Values in parentheses indicate protective potencies.

$$\text{Protective potency (\%)} = \frac{(CCl_4 \text{ group}) - (\text{Sample} + CCl_4 \text{ group})}{(CCl_4 \text{ group}) - (\text{Nontreated group})} \times 100$$

Significantly different from CCl_4-treated control group. a) $p<0.05$, b) $p<0.01$ (Student's t-test).

TABLE II. Influence of Only Antioxidants Administration on Serum Enzyme Activities

Sample	Molecular weight	Dose (mmol/kg)	GOT (IU/l)	GPT (IU/l)	LDH (IU/l)	LAP (IU/l)	T-BIL (mg/dl)	D-BIL (mg/dl)
Nontreated			130±111	18±3	434± 61	19±3	0.23±0.09	0.13±0.09
L-Ascorbic acid	198.11	1	119± 16	21±4	807±640	21±4	0.21±0.06	0.10±0.04
Sesamol	138.12	1	126± 11	22±2	835± 91	20±2	0.20±0.02	0.11±0.02
Gallic acid (hydrate)	188.14	1	97± 17	17±5	637±140	16±3	0.22±0.05	0.11±0.03
Ferulic acid	194.19	1	111± 16	16±3	817± 69	19±3	0.22±0.03	0.12±0.03
Ellagic acid (dihydrate)	338.22	0.12	117± 3	15±3	560±159	15±3	0.21±0.03	0.10±0.03
BHT	220.36	1	117± 36	17±3	448± 99	20±3	0.24±0.12	0.12±0.03
BHA	180.25	0.5	145± 69	20±5	545±381	16±3	0.25±0.03	0.14±0.05
dl-α-Tocopherol acetate	472.75	1	98± 16	19±3	462±152	20±3	0.18±0.03	0.08±0.03
Cysteine	121.20	8	124± 16	23±5	688±282	19±3	0.20±0.05	0.08±0.03
Linoleic acid	302.43	0.08	102± 33	16±3	547±151	19±3	0.19±0.02	0.09±0.03

Each value is the mean±S.D. of 3 rats.

TABLE III. Protective Effects of Antioxidants on Lipid Peroxidation Induced by CCl_4

Sample	Molecular weight	Dose (mmol/kg)	Lipid peroxide MDA nmol/g tissue	
Nontreated			133± 74	
CCl_4 control			4312±1673	
L-Ascorbic acid	198.11	1	4444±1636	(−3.2)
Isoascorbic acid	176.12	1	2403±1160[a)]	(45.7)
Sesamol	138.12	1	212± 142[b)]	(98.1)
Gallic acid (hydrate)	188.14	1	3695±1318	(14.8)
Ferulic acid	194.19	1	4001± 302	(7.4)
Ellagic acid (dihydrate)	338.22	0.12	1511±1632[a)]	(67.0)
BHT	220.36	1	3233± 234	(25.8)
BHA	180.25	0.5	3455± 540	(20.5)
dl-α-Tocopherol	430.72	1	336± 84[b)]	(95.1)
dl-α-Tocopherol acetate	472.75	1	148± 46[b)]	(99.6)
Vitamin K_1	450.71	1	2445±1008[a)]	(44.7)
Cysteamine (hydrochloride)	113.60	2	173± 98[b)]	(99.0)
Cysteine	121.20	8	104± 32[b)]	(100.7)
Linoleic acid	302.43	0.08	227± 18[a)]	(97.8)

Each value is the mean±S.D. of 4 rats (only CCl_4-treated group is 20 rats). Values in parentheses indicate protective potencies.

$$\text{Protective potency } (\%) = \frac{(CCl_4 \text{ group}) - (\text{Sample} + CCl_4 \text{ group})}{(CCl_4 \text{ group}) - (\text{Nontreated group})} \times 100$$

Significantly different from CCl_4-treated control group. a) $p<0.05$, b) $p<0.01$.

TABLE IV. Inhibition of Lipid Peroxidation of Liver Mitochondoria Fraction Induced by Ascorbic Acid Plus ADP-Fe^{3+}

Sample[a]	Molecular weight	IC_{50}[b]	
		(M)	(μg/ml)
BHA	180.25	3.94×10^{-5}	0.71
Sesamol	138.12	8.83×10^{-5}	1.22
dl-α-Tocopherol	430.72	1.61×10^{-5}	6.93
Gallic acid (hydrate)	188.14	5.43×10^{-5}	10.23
Ellagic acid (dihydrate)	338.22	6.16×10^{-4}	208.45
Cysteamine (hydrochloride)	113.60	6.35×10^{-4}	72.12
Linoleic acid	302.43	9.05×10^{-4}	273.56
Ferulic acid	194.19	9.09×10^{-4}	176.54
dl-α-Tocopherol acetate	472.75	1.17×10^{-3}	554.62
Cysteine	121.20	4.44×10^{-3}	537.98
Vitamin K_1	450.71	$>2.22\times10^{-3}$	>1000.00

a) Each sample was dissolved in Krebs–Ringer phosphate buffer or 5% Tween 20. b) 50% Inhibitory concentration.

References and Notes

1) A part of this study was published at the 109th Annual Meeting of the Pharmaceutical Society of Japan, April 1989, Nagoya
2) R.O. Recknagel, *Pharmacol. Rev.*, 19, 145 (1967)
3) E.A. Glende, A.M. Hruszkewycz, R.O. Recknael, *Biochem. Pharmacol.*, 25, 2163 (1976)
4) E.C. Ferreyra, J.A. Castro, M.I.D. Gomez, N.D. Acosta, C.R. Castro, O.M. Fenos, *Toxicol. Appl. Pharmacol.*, 32, 504 (1975)
5) R.J. Ruch, J.E. Klaauning, N.E. Shultz, *Envir. Health Perspect.*, 69, 301 (1986)
6) A.K. Ghoshal, *Exp. Mol. Pathol.*, 25, 202 (1976)
7) A.L. Tapple, *Ann. N. Y. Acad. Sci.*, 355, 18 (1980)
8) T. Yoshikawa, Y. Furukawa, M. Murakami, *Digestion*, 25, 222 (1982)
9) S. Nagase, H. Tanaka, "Clinical, Biochemical Data on Experimental Animals - Relation with Pathohistological Image", Soft Science, 220-230 (1976)
10) S. Ohta, M. Kumasaka, M. Shinoda, *YAKUSHI*, 105, 866 (1985)
11) A. Karmen, F. Wroblevski, J.S. LaDue, *J. Clin. Invest.*, 34, 131 (1955)
12) W.E.C. Wacker, D.D. Ulmer, B.L.C. Vallee, *New England. J. Med.*, 255, 449 (1956)
13) W. Thefeld, H. Hoffmeister, E.W. Busch, P.U. Koller, J. Vollmar, *Deut. Med. Wochenschr*, 99, 343 (1974)
14) L. Jendrassik, R.A. Cleghorm, *Biochem. Z.*, 289, 1 (1936)
15) H. Tanizawa, Y. Sazuka, Y. Takino, *Chem. Pharm. Bull.*, 29, 2910 (1981)
16) H. Tanizawa, Y. Sazuka, A. Komatsu, S. Toda, Y. Takino, *Chem. Pharm. Bull.*, 31, 4139 (1983)
17) T. Okuda, Y. Kimura, T. Yoshida, T. Hatano, H. Okuda, S. Arichi, *Chem. Pharm. Bull.*, 31, 1625 (1983)
18) E.C. Ferreyra, O.M. Fenos, A.S. Bernacchi, C.R. Castro, J.A. Castro, *Toxicol. Appl. Pharmacol.*, 42, 513 (1977)
19) A.W. Wood, M.T. Huang, R.L. Chang, H.L. Newmark, R.E. Lehr, H. Yagi, J.M. Sayer, D.M. Jerina, A.H. Conney, *Proc. Natl, Acad. Sci. U.S.A.*, 79, 5513 (1982)

20) J.M. Sayer, H. Yagi, A.W. Wood, A.H. Conney, D.M. Jerina, *J. Am. Chem. Soc.*, **104**, 5562 (1982)
21) H. Mukhtal, M. Das, B.J. Del Tito, Jr., D. Bickers, *Biochem. Biophys. Res. Commun.*, **119**, 751 (1984)
22) Y. Fujita, I. Uehara, Y. Morimoto, M. Nakajima, C. Hatano, T. Okuda, *YAKUSHI*, **108**, 129 (1988)